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A complete YAC (yeast artificial chromosome) contig is being constructed across a defined region in 11p15.5 associated with breast cancer using our chromosome 11 YAC library. Candidate genes will be identified by exon amplification and cDNA enrichment. Chromosome rearrangements in this region will be utilized for mapping and isolation of tumor suppressor genes. Breast cancer tissue will be examined by Southern blot and ribonuclease protection analysis to identify possible tumor suppressor genes. Candidate genes will be analyzed using single strand conformational analysis (SSCA) and chemical mismatch cleavage (CMC) to identify mutations. These studies should provide insights into the genetic mechanisms involved in breast cancer development and diagnostic tools for subclassifying breast tumors with alterations at 11p15 and, therefore, be of prognostic value.

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5. INTRODUCTION

Frequent loss of genetic material in chromosome band 11p15.5, manifested as loss of heterozygosity (LOH) has been observed in breast cancer patients. The existence of a growth or tumor suppressor gene is also suggested by chromosome-fragment mediated gene transfer studies demonstrating that loci between D11S12 and IGF2 exhibit growth-arrest in vivo or tumor suppression in nude mice. It is unclear whether distinct tumor suppressor genes are lost in the breast cancer-associated chromosome region or whether a more general suppressor locus (analogous to p16 or p53) exist in 11p15.5 which; when inactivated, contributes to tumor progress in a wide range of malignancies. Tumors associated with the overgrowth condition Beckwith-Wiedemann syndrome (BWS) also show LOH in 11p15.5. Furthermore, rare BWS patients have constitutional chromosome rearrangements with breakpoints affecting the same regions of 11p15 showing LOH. Chromosome translocations and inversions in BWS patients may be directly disrupting tumor suppressor or other growth regulating genes. On the assumption that these breakpoints are affecting genetic loci, important in tumor suppression, we are examining several such chromosome aberrations to identify genes or "controlling" regions affected by the rearrangements.

SPECIFIC AIMS

1. To construct a YAC-based clone contig map of the breast cancer associated 11p15.5 region.

2. Using fluorescence in situ hybridization (FISH), YACs that cross chromosome breakpoints in this region are being identified.

3. Identify cognate cosmid clones for the YAC clones that cross breakpoints. P1 based artificial chromosomes are being used to supplement the chromosome 11 cosmid library.

4. Identify and characterize transcribed sequences in the breakpoint region by exon amplification and/or cDNA selection.

5. Characterize genes disrupted by breakpoints.

6. Assess transcribed sequences as candidate disease genes by mutation analysis in patients and tumors.

6. BODY - RESULTS

A. YAC Contig Assembly

A 4X chromosome 11 specific YAC library (Qin et al., 1993) has been the main source of clones due to the low incidence of chimeric clones and the library's small size (1824

clones), which facilitates screening procedures. Since all but ~100 clones have already been assembled into contigs in the 1 Mb range (Qin et al., 1995), subsequent screening with regionally mapped loci is likely to identify relatively large contigs (rather than 2 or 3 overlapping YACs) resulting in a straightforward process to join up initial contigs by an end-walking approach.

Several YAC contigs have already been identified in the critical interval between HBB locus to the telomere see (Fig. 1). The chromosome 11 YAC library was screened primarily by Alu-PCR hybridization or by PCR. A 2 Mb contig linking HBB and RPMI (Fig. 1) has already been assembled. Hybridization of Alu-PCR products derived from cosmids cCK2-2, cCI11-565 (D11S601) and cCI11-555 (D11S724) have also identified small contigs in the region just centromeric to IGF2. In addition, YAC contigs encompassing HRAS/RNH and the telomere have been established (Fig. 1). The ends from terminal YACs in each of the assembled contigs have been isolated and cloned using a modification of the PCR method described by Kere et al. (1992). Sequences derived from these clones will be used to generate new STS markers to rescreen the chromosome 11 YAC library or additional libraries to join existing contigs by chromosome walking. This approach has proven successful in extending the contigs identified with one of the flanking markers of the USH1C locus (Higgins et al., 1995).

B. Additional YAC Libraries

In the event that the chromosome 11 specific YAC library does not contain clones for a particular locus, DNA pools for two other YAC libraries are currently being used in the lab (CEPH-A [Research Genetics] and the ICI YAC libraries [UK Human Genome Project Resource]). Following PCR screening of these pools, individual clones can be purchased.

C. P1-artificial Chromosome (PAC) Library

We have obtained the human PAC library (Ioannou et al., 1994) made in P. de Jong's laboratory (in this Department), as well as high density filters and bacterial cell pools for screening by hybridization and PCR, respectively. While the average size insert of 130 kb is smaller than YACs, it is possible that the PAC vector/host system may allow the cloning of human sequences too unstable in yeast to be found in YAC libraries. This PAC library has been used to construct a 350 kb PAC clone contig extending from D11S517 to D11S1 (Fig. 1). This library will also be used to convert YAC contigs into PACs for gene isolation as described below.

D. Bacterial Artificial Chromosome (BAC) Library

As an additional alternative to YAC libraries, we have screened the 2-3 fold human BAC library (average size 120 kb) (Research Genetics) and detected two BAC clones in the library that contain H19, a locus for which we have been unable to identify YACs in either the chromosome 11 library or CEPH-A YAC libraries. Thus, the BAC and PAC libraries appear to be useful to "fill" gaps between YAC contigs in the region of interest.

E. Conversion of YAC Contigs Into PACs

Because of the difficulty in isolating sufficient high quality YAC DNA for gene isolation techniques, we are "converting" YAC contigs into PAC clones by hybridization screening of high density filters. To test the efficiency of this approach, we have generated Alu-PCR products from 13 overlapping YACs comprising a "shortest path" through two contigs (approximately 2 Mb and 0.7 Mb) (Higgins et al., 1995). These amplification products were hybridized to seven high density filters (~ 18,000 clones each). A secondary filter containing 91 possible positive clones were hybridized with Alu-PCR products from the YAC clones identifying 25 strongly hybridizing PAC clones. Since the YACs used in this analysis overlap to some extent, 25 PAC clones with an average insert size of 130 kb is what one might expect to identify in the 3.0-fold PAC library. The secondary screening procedure not only identifies true positive clones but simultaneously helps to confirm YAC overlaps (since overlappping YACs might be expected to detect PAC clones in common) and establishes a cursory order of the PACs with respect to the YAC physical map. Similar results have been obtained in the conversion of several other YAC contigs, and we anticipate comparable success in converting YAC contigs in the asynchronously replicating region in 11p15.5.

F. Isolation of Novel Genes by Direct cDNA Selection

Positional cloning has been greatly facilitated by the development of novel methods to identify transcribed sequences from complex DNA sources. Variations on one such procedure, cDNA selection, have been developed in several laboratories (Lovett et al., 1991; Parimoo et al., 1991; Korn et al., 1992; Rommens et al., 1993) and have been responsible for identifying large numbers of novel genes over hundreds of kilobases of DNA (Morgan et al., 1992; Rommens et al., 1993; Lovett, 1994).

Despite its relative technical simplicity, the method of Rommens et al. (1993) has proven extremely effective and has been used in the recent cloning of the genes responsible for Wilson disease (Bull et al., 1993) and one form of familial Alzheimer's disease (Sherrington et al., 1995). Following several visits to Dr. Rommens lab (Hospital for Sick Children, Toronto), our lab has now isolated four new transcripts in 11p15.5 which are currently being characterized.

1. Generation of cDNA Probes

The original versions of the cDNA selection procedure used PCR-amplified inserts from cDNA libraries as probes (Lovett et al., 1991; Korn et al., 1992). Since cloning bias is inherent to any library, and some mRNA species may have been under-represented in these probes, Rommens et al. (1993) implemented the use of PCR-amplified primary uncloned cDNA as a probe for cDNA selection.

Briefly, first-strand cDNA synthesis was carried out with each purchased RNA sample (kidney, fetal kidney and liver, testes, adult and fetal brain, frontal cortex, adrenal gland, skeletal

muscle, placenta) using a modified random primer (RXG dN6, where RXG is a linker containing EcoRI, XhoI, and BglII restriction sites, and serves as a binding size for PCR primers) and M-MLV reverse transcriptase (RT) (Rommens et al., 1994). Following "tailing" with dATP by terminal transferase, second-strand cDNA synthesis was carried out by two rounds of extension with Taq polymerase and a modified oligo(dT) primer [RXG$(dT)_{12}$]. The extensions were carried out in PCR buffer (10 mM Tris-Cl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, pH8.3) and included an initial denaturation at 92°C for 4 min, followed by two cycles of denaturation at 92°C for 45 sec, annealing at 37°C for 45 sec, and extension at 72°C for 150 sec). Each cDNA pool was then expanded by PCR using RXG primers (corresponding to the restriction site containing linker) for 18 cycles. The second strand synthesis and PCR expansion were carried out consecutively using linked thermocycler programs. The reaction mix contained both RXG and RXG$(dT)_{12}$ primers at a 15:1 ratio.

2. Preparation of DNA Target Filters

Several cosmid and YAC clones were used in our initial attempt to isolate new genes using cDNA selection. Two μg of each cosmid DNA, either alone or together with other cosmids, was electrophoresed into a 0.7% agarose gel and a small (3mm x 10mm) gel slice containing the DNA was removed. For YACs, ten 1cm plugs (approximately 10 μg total yeast plus YAC DNA) were electrophoresed in a preparative CHEF gel (BioRad; 1% agarose, 0.5 x TBE, 60 sec switch time for 24 at 200V) and gel slices containing the YACs were removed. Cosmid DNA (either single clones or pools of clones) and gel isolated YAC DNA was immobilized onto small strips (3mm x 10 mm) by a Southern blotting procedure and then hybridized over six days with a pool of amplified cDNA fragments made from poly$(A)^+$ RNA from eight different tissues. The use of such a complex hybridization mixture allows the "scanning" of transcripts from many tissue sources simultaneously without apparent loss in sensitivity. After washing the filters, elution and PCR, specifically bound material was cloned into Bluescript (Stratagene), and a number of the unique sequence clones analyzed.

3. Four Novel Transcripts in 11p15.5

A positive control cosmid, which contains the C4 cathepsin D (CTSD) gene, was included in one of the cosmid pools. Several cDNA fragment clones that mapped back to cosmid C4 were sequenced and found to encode for CTSD, indicating that the procedure had worked. Another cosmid (N60D9), which maps just telomeric to the MUC2 (mucin 2) gene, selected several cDNA clones that showed cross-species conservation, and detected a 2.2 kb transcript by Northern analysis in all tissues examined. Sequence analysis and subsequent BLAST searches of sequence databases indicated a similarity (~80%) but non-identity to MUC2, and suggest that these cDNA clones represent a novel member of the mucin gene family which maps very close to MUC2 and MUC5.

Cosmid cI11-555 (D11S724), which was found to span a BWS inv(11) breakpoint (Sait et al., 1994), was also used in the cDNA selection protocol. Thirteen unique cDNA clones

selected by this cosmid were sequenced and grouped into five sequence contigs. Despite showing cross hybridization to other mammalian species, homology searches using BLAST failed to find any significant matches with known genes. However, the majority of these clones detected highly significant matches with randomly selected cDNA clones in the dbEST database (Expressed Sequence Tag database). Only one of the clones contained a significant open reading frame (ORF). Similar "non-coding" transcripts have been found in two other imprinted regions in humans, the IPW and PAR genes in the PWS/AS region on chromosome 15 (Wevrick et al., 1994; Nakao et al., 1994), and the XIST gene on the X-chromosome (Brown et al., 1991). On Northern blots, two of the clones selected with c555 detect 4.2 kb and 7.5 kb transcripts in adult brain and kidney, respectively. Representative clones from three other sequence contigs detect a complex pattern of multiple transcripts in several tissues suggesting that they may be part of the same gene. The relationship between these clones and those identified in dbEST is ongoing.

Most recently, we have used a YAC just centromeric of cCI11-555 to select a cDNA clone, 5-111, which demonstrated cross-species hybridization on Zoo blots and detected a 2.8 kb transcript on a Northern blot in all the same RNA samples, as well as in fetal brain, lung, liver, and kidney. This clone was used to screen cDNA libraries prepared from fetal brain and Caco-2, an intestinal cell line. A total of 16 clones hybridizing to 5-111 were identified. Based on the size of the mRNA detected on Northern blots, two of these are likely to be full length. Furthermore, restriction enzyme mapping suggests that there are two classes of clones differing by 500 bp in the central restriction fragments. These clones may be a result of alternative splicing. Preliminary sequence analysis suggest the presence of a substantial ORF, and BLAST searches indicate a high degree of homology to a nucleosome assembly protein. At present, we are doing further sequencing of these cDNA clones to assemble the full length sequence.

G. Localization of Tumor Associated Rearrangement Breakpoints

Part of the long-range restriction map (Higgins et al., 1994) has been used to precisely map chromosome rearrangement breakpoints from three BWS patients and a rhabdoid tumor. These breakpoints may be: (i) disrupting a tumor suppressor or other growth regulating gene; (ii) affecting nearby genes by altering local regulatory elements; or (iii) altering the normal genomic imprinting of the region. All four breakpoints were located in a 250-675 kb interval distal to D11S679 and at least 270 kb proximal to IGF2 and H19 (Sait et al., 1994). Pulsed-field gel analysis indicated that none of these breakpoints directly disrupted IGF2 or H19, two candidate genes for BWS. As a result of this work, cosmid cCI11-555 (D11S724) was shown to span one of the BWS rearrangement breakpoints (Sait et al., 1994; see Fig. 1). This cosmid has since been used to select novel cDNA clones, as well as identify YACs corresponding to this locus (Fig. 1). FISH analysis has shown that one of these YACs spans two of the three BWS breakpoints (inv[11], t[11;16]) examined, thereby locating them together within the length of the YAC (225 kb).

7. CONCLUSIONS

A physical map across the 11p15.5 region is thought to contain a breast cancer gene. Studies have begun to isolate candidate genes and to analyze patients for alterations in the region. Our immediate future plans follow.

A. Saturation cloning of transcribed sequences in the vicinity of the four breakpoints, including the isolation of full length cDNA clones.

B. Characterization of full length cDNA clones by Northern blot, RT-PCR analysis and sequencing.

C. Identification of genes that are disrupted or whose expression profile is altered by the breakpoints.

D. Analysis of patients and tumors for mutations.

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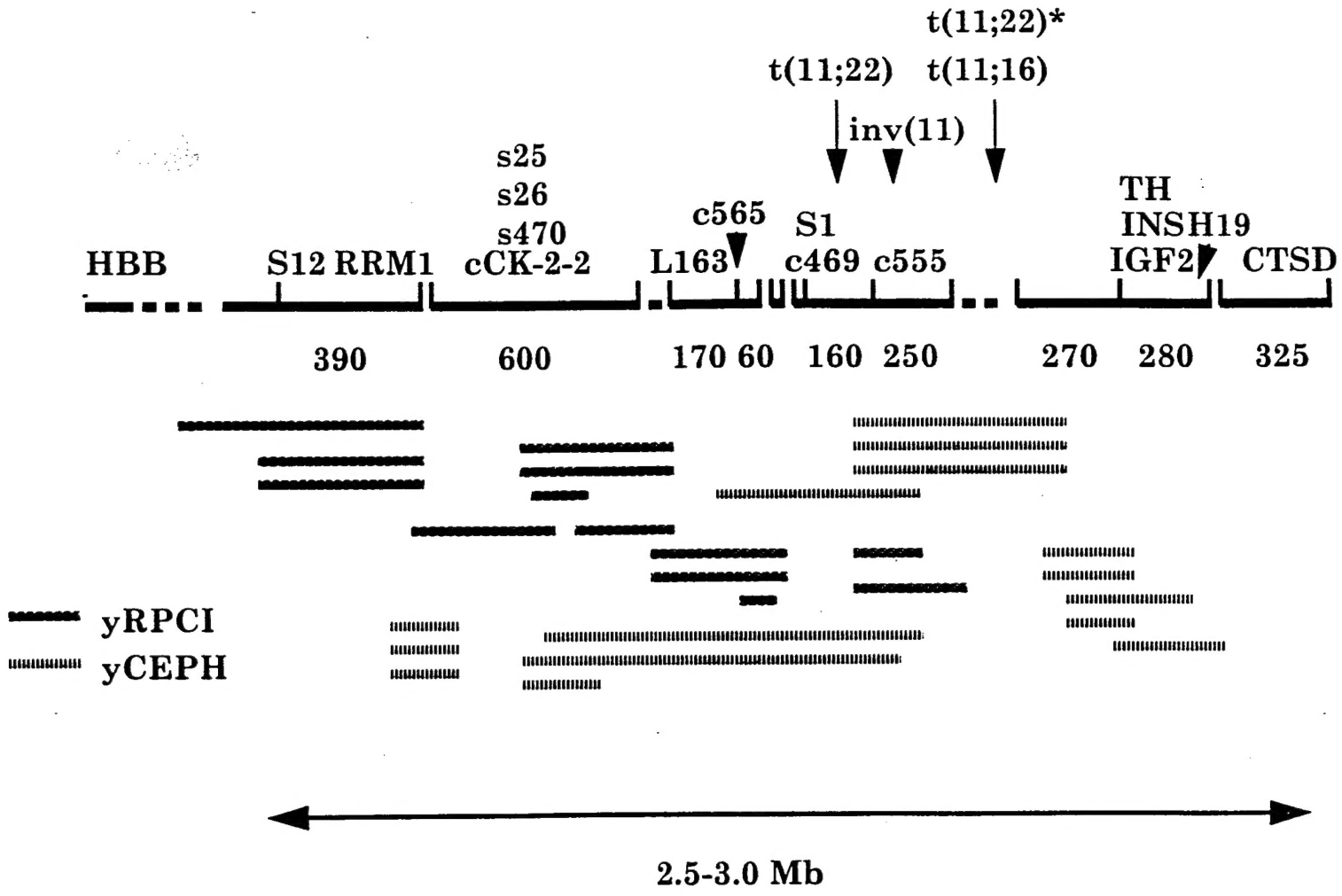

Fig. 1. Physical map of region associated with breast cancer. Solid bars represent the genomic NotI map between HBB and CTSD. The positions of the three BWS and rhabdoid tumor (*) rearrangement breakpoints are shown above the map. A subset of YAC clones covering the region is shown below the map.